

FINAL TECHNICAL REPORT

Submitted to

Department of Army
Army Research Office
4300 South Miami Blvd.
Research Triangle Park, NC 27709-2211
Tel.: (919) 549-4228

Project Number: Proposal No. P-32446-LS-ISP
Cooperative Agreement No. DAAH04-93-2-0004
WES/HBCU/MIS Collaborative Proposal No. 301

Army Technical Officer: Dr. D. Gunnison, WES, Vicksburg, MS

Project Title: Search for genes having TNT degrading capability for
bioremediation

Start Date: October 25, 1993

Completion Date: September 18, 1995

End of No-Cost Extension: September 18, 1996

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204 Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 11-22-96		3. REPORT TYPE AND DATES COVERED 10-25-93 to 9-18-96	
4. TITLE AND SUBTITLE Search for Genes having TNT degrading capability for bioremediation				5. FUNDING NUMBERS DAAH04-93-2-0004	
6. AUTHOR(S) Sisir K. Dutta, Ph.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Department of Biology Howard University Washington, D.C. 20059				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research Office P. O. Box 12211 Research Triangle Park, NC 27709-2211				10. SPONSORING / MONITORING AGENCY REPORT NUMBER ARL 32446.1-LS-ISP	
11. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Several achievements are made through this initial collaborative project. These are: 1) creation of enormous capability at this HBCU in enhancing this research; 2) extensive studies on identification of TNT biotransformation products in several bacterial species and one fungal species; 3) identification of several genes which are capable of catalyzing degradation of metabolites of TNT in several microbial species, particularly in the fungus <i>P. chrysosporium</i> ; 4) isolation and DNA sequence of these genes for probe development; and 5) PCR amplification of 2,4-DNT gene using <i>P. chrysosporium</i> genomic DNA as template; 6) DNA:DNA hybridization with ³² P labeled 2,4-DNT dioxygenase gene (used as probe) to anneal with <i>P. chrysosporium</i> genomic DNAs; and 7) selection of proper combination of mixed genotypes of bacteria for very efficient degradation of TNT.					
14. SUBJECT TERMS				15. NUMBER OF PAGES 18	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL		

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Contents

		Page #
I.	Introduction	3
II.	Nature of Collaboration	3
III.	Objectives	3
IV.	TNT Research Capabilities Created at this HBCU New Physical Instrumentation Facilities Created Students and Personnel Trained	3
V.	Culture Collections and Their Roles in TNT Disappearance Bacterial and fungal activities for cell growth, peroxidase activities and biotransformation Role of peroxidase gene in TNT disappearance Biotransformation studies (HPLC analyses) ¹⁴ C-TNT mass-balance studies for estimating biodegradation	4
VI.	Search for Genes Involved in TNT disappearance Different Approaches for searching genes. Role of mixed cultures containing known genes Homology studies of the 2,4-DNT genes Search for Am-DNT genes from highly contaminated soil with the explosive TNT	13
VII.	Ancillary Studies: Genotoxicity of TNT Metabolites.	16
VIII.	Summary of Achievements.	16
IX.	References	17
X.	Presentations of Papers from this Project at Various Meetings.	18
XI.	Papers Under Preparation.	18

I. INTRODUCTION

Bioremediation has been proposed as a strategy to clean TNT contaminated sites. Some bacterial species, particularly *Pseudomonas* are capable of growing in TNT concentration that are fatal to fungi. For example, if the TNT concentration is greater than 50 ppm the fungus *Phanerochaete chrysosporium* is killed, however this fungus can mineralize TNT in pure culture though little is known about the pathway and genes involved in the fungus.

The primary goal of this project is to create a microbial system capable of mineralizing TNT in sites with high TNT levels. Apparently a cluster of genes are involved in TNT degradation. Only a few of these genes have been characterized. TNT is non-specifically transformed to isomers of mono-aminodinitrotoluene (Am-DNT). The genes involved in DNT (Suen and Spain, 1993) and toluene mineralizations are well characterized (Fanklin et al., 1981) but the genes which degrade Am-DNT are unknown.

II. NATURE OF COLLABORATION

This initial project was the result of a Broad Agency Announcement from Army with three primary goals: 1) Build up a collaboration with the ongoing activities of an Army Laboratory. This was possible due to creation of collaboration (mostly as advisor) with Dr. Douglas Gunnison, Sr. Microbiologist of Army Corps of Engineers at WES. 2) Provide help to a historically black college or university (HBCU) in building up the capability in bioremediation research. 3) Identify genes which code for TNT degradation in bacterial and fungal organisms.

III. OBJECTIVES

The specific technical objectives of this work unit are as follows: (1) Enrichment and isolation of TNT degrading microbial cultures; (2) Characterization of gene(s) involved in the TNT degradation mechanism. These degradation genes will be characterized in both the white rot fungus and bacterial isolates.

IV. TNT RESEARCH CAPABILITIES CREATED AT THIS HBCU

Howard University, as part of its commitments to this collaboration has invested \$60,000 in a new plant and soil research laboratory to work exclusively with TNT and related toxic chemicals. These facilities include: new HPLC (high performance liquid chromatography) system, two plant growth chambers, facilities for anaerobic microbiology and PCR..

Following pre- and post-doctoral students trained in molecular biology techniques, mostly on voluntary basis except a few who received remuneration as identified below:

Undergraduate Students:

Daphne Jones
Nicole R. Holmes
Erik Watson
Benjamin Dyson Jr. (paid from Army project)
Adebisi Ajala
Khaled S. Mirza (paid from Army project)
Ike Anucha

Graduate Students:

Jacintha D. Elder
Nisha Isaac
Michelle M. Jackson (paid from Army project)
LaShawn R. Drew
Hirendra N. Banerjee (partly paid from Army project)
Gregory Davenport
James Stewart
Anhtuan L. Truong
Gail Hollowell (paid from Army project)

Post-Doctoral Fellow and/or Collaborators:

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 P.C. Das
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 V. Rangaswamy
 David Powell
 Li-Hua Hou (paid from Army project)
 William Gillete
 Michael M. LaMontagne
 L.D. Kuykendall

V. CULTURE COLLECTION AND THEIR ROLES IN TNT DISAPPEARANCE

Bacterial and Fungal Activities for Cell Growth, Peroxidase Activities and Biotransformation:

Bacteria and fungal cultures that grow in presence of TNT are listed in Table 1. In bacterial biomass (Figure 1 and Table 2), protein (Table 3), and NO₂ (Table 4) levels increased in the presence of TNT bacterial cultures. HPLC analysis indicates a non-specific increase in Am-DNT (Figure 2) in all cultures tested. The mechanism responsible for the biotransformation of TNT to Am-DNT has not been elucidated. In fungal culture *Phanerochaete chrysosporium* TNT decreased biomass but the culture reduced TNT concentrations (Table 2).

P. chrysosporium thrives on woody substrate where it effectively degrades lignin. The lignin degrading system involves peroxidase or commonly referred to as lignin peroxidase (LiP) and manganese peroxidase (MnP), as well as H₂O₂-generating peroxidase (Gold et al., 1989; Kirk and Farrell, 1987). Under ligninolytic conditions this fungus can degrade a variety of pollutants such as PCB (polychlorobenzene), 1,1,1-trichloro-2-bis(4-chlorophenyl) ethane (DDT), phenanthrene, 2,4,5-trichlorophenoxyacetic acid, BTEX (benzene, toluene, ethyl benzene, and xylenes) and 2,4,6-TNT (Kohler et al., 1988; Sutherland et al., 1991; Yadav and Reddy, 1992; Spiker et al., 1992). This fungus does not express the extracellular peroxidase under non-ligninolytic conditions. It was essential for us to know the role of peroxidase enzyme in degrading TNT and its metabolites. We have compared the ability of *P. chrysosporium* to degrade TNT, 2,4-DNT and 2-Am-DNT under ligninolytic and non-ligninolytic conditions. The degradation of 2,4,6-TNT by this fungus has already been reported (Michels and Gottschalk, 1995).

Peroxidase activities in *P. chrysosporium* cells are seen only under lignolytic (low nitrogen) condition (Fernando et al., 1990; Valli et al., 1992). ¹⁴C-TNT disappearance (Figure 2) and HPLC analysis indicate TNT is degraded under non-ligninolytic condition. These data provide insight, not reported before, with respect 2,4-DNT disappearance, was not dependent on peroxidase enzyme, suggesting that this fungus has genes for 2,4-DNT degradation (Tables 5 and 6).

In this study we have shown that bacterial cultures which were isolated from 2,4,6-trinitrotoluene (TNT) contaminated soil can biotransform TNT by non-specific reduction of NO₂ and form monoaminodinitrotoluene (Am-DNT). This non-specific NO₂ reduction does not happen in killed (inactivated) cells, nor TNT is absorbed by cells. This transformation to Am-DNT is facilitated by the presence of the cometabolite 0.5% NaOAc. When 0.5% NaOAc was omitted, cells in all cultures grew very slowly (OD/600 nm 0.2-0.5). In other laboratories they have used yeast (200 mg/L) extract or glucose. This shows for initial growth, some push from co-metabolites are needed for degradation of TNT and DNT. The ability to degrade Am-DNT appears to be plasmid based. *P. putida* strain 2015 cannot degrade Am-DNT without the presence of the plasmid pWWO (Figure 3).

TABLE 1 . List of cultures used and presence of plasmids in cells.

Names	OD/600 nm					Presence of plasmid	Source
	Control	Killed cells	Days grown				
			1	5			
<i>Pseudomonas aeruginosa</i>	0.00	0.019	1.117	0.086	+		Isolated by Dr. Gunnison
<i>P. corrugata</i>	0.00	0.015	1.259	1.123	+		“
<i>P. fluorescens</i>	0.00	0.007	1.165	0.806	+		“
<i>P. nitroreducens</i>	0.00	0.021	1.212	0.742	+		“
<i>P. putida</i> (2015)	0.00	0.010	1.006	0.971	None		Jeff Kam, USDA
<i>P. putida</i> paW1 with plasmid pWWO	0.00	0.015	1.424	0.738	+		R. Olsen, Univ. of Michigan, MI
<i>P. putida</i> PPO200 paW1 cured of plasmid pWWO							R. Olsen, Univ. of Michigan, MI
<i>Klebsiella pneumoniae</i>	0.00	0.011	1.238	0.563	+		Isolated by Dr. Gunnison
<i>Enterobacter gergoviae</i>	0.00	0.009	1.03	0.918	+		“
<i>Xanthomonas maltophilia</i>	0.00	0.020	1.035	1.097	+		“
<i>Burkholderia</i> sp. DNT	0.00	0.021	1.560	1.269	+		J. Spain, Tyndall Airforce Base, FL
<i>Phanerochaete chrysosporium</i> BKM-F-1767	0.00	-	Dry wt (see Table 2)		None		C.A. Reddy, Michigan State Univ., MI

Control, no cell; killed cell, cells were boiled for 10 minutes before inoculation.

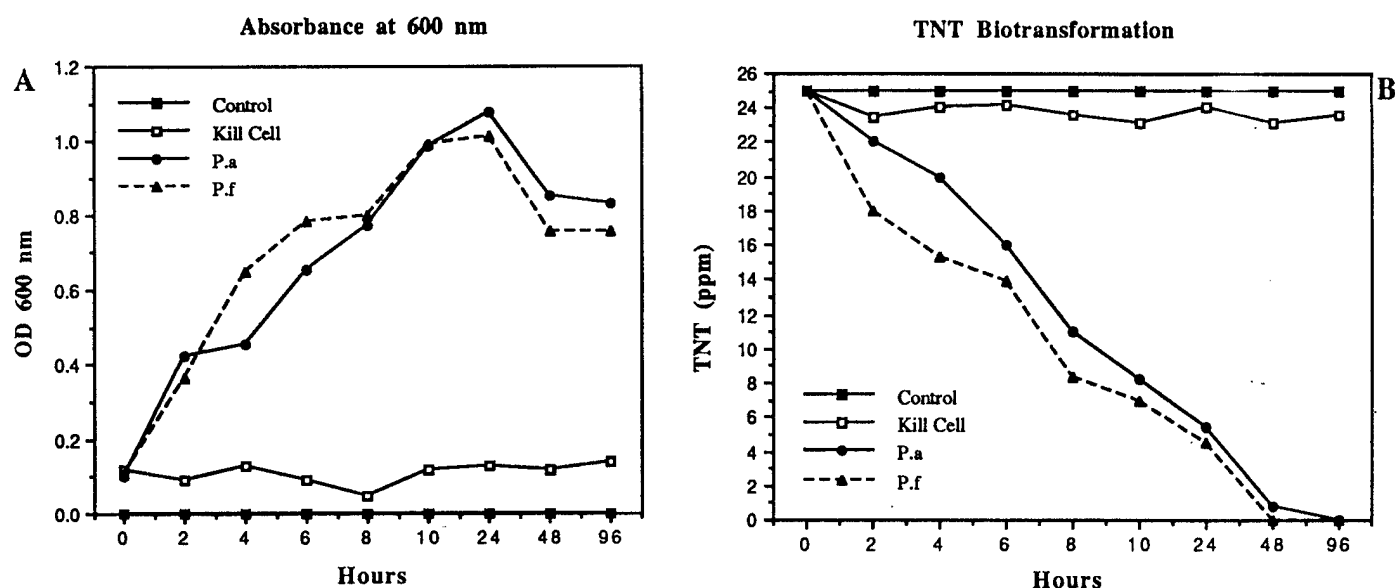


TABLE 2: Growth Pattern of the Fungus *Phanerochaete chrysosporium* Under Different Media^a.

Dry Weights of Mycelia (mg)			
Days of Growth	Without TNT ^b	With TNT ^c	% TNT Disappearance
1	13.7 ± 0.2	12.5 ± 0.4	34
2	14.6 ± 0.3	13.2 ± 0.3	81
3	14.9 ± 0.3	13.8 ± 0.4	95
7	16.2 ± 0.4	14.9 ± 0.6	100

a, Values are mean + s.d. for 3 replicate cultures.

b, Culture flask contained minimal salt medium without any carbon and nitrogen source. Supplemented with 22 mM glucose, 0.48 mM ammonium tartrate, 0.1 mM Tween-80, and 0.07 mM veratryl alcohol.

c, Culture flask contained minimal salt medium. In addition it contained 80% reduced amount 4.4 mM glucose, 0.09 mM ammonium tartrate, 0.02 mM Tween-80, and 25 ppm TNT.

Very minimal (less than 1 mg) growths were detected when cultures were without TNT but with the same amount of cometabolites. These data confirm that this fungal strain is utilizing TNT as the sole source of carbon and nitrogen.

TABLE 3: Composite data of assays for protein by different 5-days old bacterial cultures.

Names	Protein Assays			
	Without TNT		With TNT (25 ppm)	
	OD/595 nm	µg/ml	OD/595 nm	µg/ml protein
<i>Enterobacter gergoviae</i>	0.209	5.6	0.525	15.0
<i>Pseudomonas aeruginosa</i>	0.176	5.0	0.382	10.9
<i>P. fluorescens</i>	0.266	7.6	0.432	12.3
<i>P. nitroreducens</i>	0.184	5.3	0.279	7.8
<i>P. putida</i> PaW1 with plasmid pWWO	0.321	9.1	0.552	14.8
<i>P. putida</i> PPO200 PaW1 cured plasmid pWWO				
<i>Pseudomonas corrugata</i>	0.170	4.9	0.180	5.1
<i>Klebsiella pneumoniae</i>	0.216	6.1	0.469	13.4
<i>Xanthomonas maltophilia</i>	0.136	3.9	0.655	18.7
<i>Burkholderia</i> sp. DNT	0.189	5.4	0.249	7.1
<i>Pseudomonas</i> 2015	0.084	2.4	0.094	2.7

Total proteins in supernatants of each cultures were estimated using Bradford method (Bradford, 1976). Similar studies done in 1 and 2 days old culture did not show significant difference in color developments with or without TNT in the growth medium. Protein was measured by the method of Bradford using BSA as a standard.

TABLE 4: Composite data of several assays for NO₂⁻ release by different 5-days old bacterial cultures.

Names	NO ₂ ⁻ Release Assays			
	Without TNT		With TNT	
	Color (before assays)	OD/540 nm µg/ml	Color (before assays)	OD/540 nm µg/ml
<i>Enterobacter gergoviae</i>	Pale Brown	0.097	Bright yellow	0.186
<i>Pseudomonas aeruginosa</i>	Green	0.277	Light yellow	0.386
<i>P. fluorescens</i>	Light green	0.225	Light green	0.554
<i>P. nitroreducens</i>	Yellow	0.090	Bright yellow	0.362
<i>P. corrugata</i>	Green	0.173	Green	0.137
<i>P. putida</i> PaW1 with plasmid				
pWWO	White	0.023	Yellow	0.199
<i>Klebsiella pneumoniae</i>	Deep green	0.142	Deep green	0.297
<i>Xanthomonas maltophilia</i>	Yellow	0.026	Bright yellow	0.409
<i>Burkholderia</i> sp. DNT	White	0.019	White	0.271
<i>Pseudomonas</i> 2015	White	0.070	White	0.259

NO₂⁻ release assays were similarly tested in the supernatant only of the growth medium. Similar studies done in 1 and 2 days old culture did not show significant difference in color developments in with or without TNT in the growth medium however, on 5th day old bacterial culture media did show distinct color differences. NO₂⁻ release assay using KNO₃ as a standard was done as described (Joy and Hageman, 1966). Data shown here were with culture medium after cells were discarded by centrifugation.

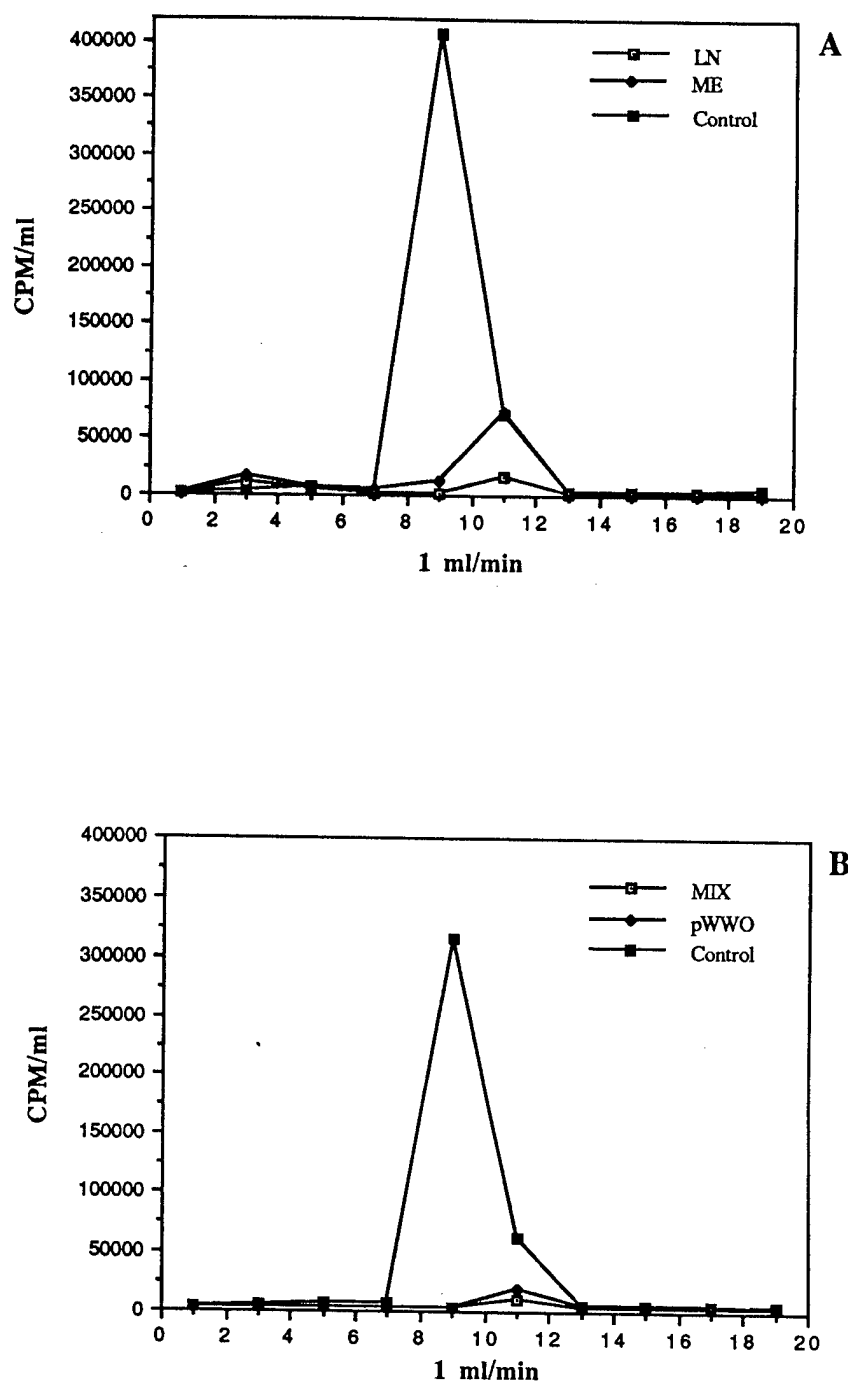


FIGURE 2: (A) HPLC elution profile of methylene chloride extract of [14 C]-TNT control, low-nitrogen, and malt extract culture of *P. chrysosporium* that was incubated with [14 C]-TNT (0.5 μ Ci) in 50 ml growth medium. (B) HPLC elution profile of a methylene chloride extract of samples (bacterial culture). 0.5 μ Ci (14 C) TNT in 25 ml growth medium. 2 ml fraction (1 ml/min) collected. Mixed cultures containing TOL gene (*P. putida* pWWO) + 2,4-DNT gene (*Burkholderia* sp.) was used. In both instances (bacteria and fungus) TNT disappeared and Am-DNT increased.

TABLE 5: ^{14}C -TNT Remaining in Two Growth Conditions of the Fungus *P. chrysosporium*.

	Day	Counts per minute (cpm)	
		Malt Extract	Low Nitrogen
Culture medium	0	725,400	704,700
Culture medium	13	308,320	352,700
Percentage		42.5%	50.0%

The actual ^{14}C -TNT cpm applied in the growth medium on the 3rd day after inoculation of spores which is regarded as "day 0" here. Similarly ^{14}C -TNT cpm were taken from the growth conditions after 13 days.

TABLE 6: HPLC Analysis of TNT Under Two Growth Conditions Using *P. chrysosporium*.

PPM of TNT Remaining		
Day	Malt Extract (25 ppm)	Low Nitrogen (ppm)
0	25	25
7	3.4	0
Biotransformation Percentage	86%	100%

Data shown above are actual concentration of TNT in ppm based on areas at seen in the HPLC (high performance liquid chromatography) data sheet at the same retention time 9.0 ± 0.5 minutes using supplecosilTM LC18 column size 150x4.6 mm (Hewlett Packard HPLC System Model # HP1050). Mobile phase 55:46 (water:methanol). Flow rate 1 ml/min. Room temperature and wave length 254 nm.

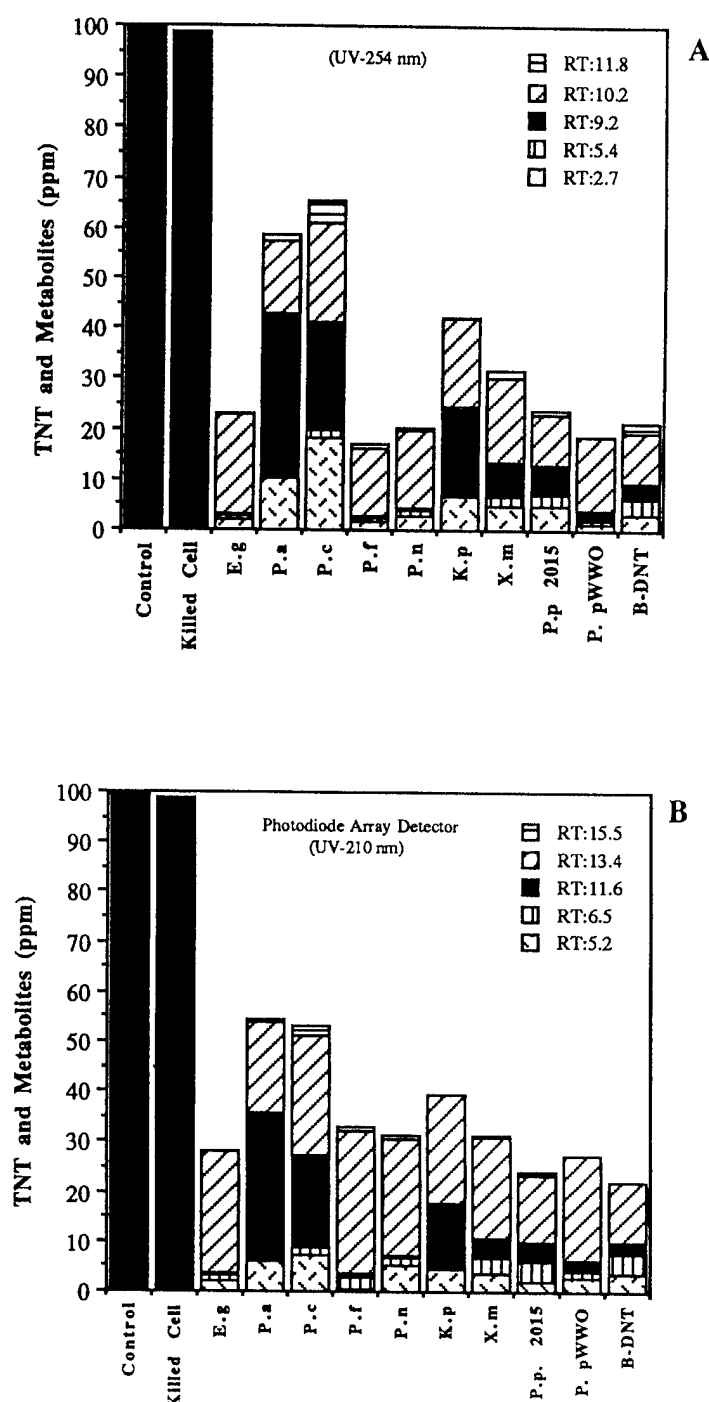


FIGURE 3: Composite histograms of TNT (100 ppm) biotransformation products. E.g., *Enterobacter gergoviae*; P.a., *Pseudomonas aeruginosa*; P.c., *P. corrugata*; P.f., *P. fluorescens*; P.n., *P. nitroreducens*; K.p., *Klebsiella pneumoniae*; X.m., *Xanthomonas maltophillia*; P.p. 2015, *Pseudomonas putida* 2015; P. pWWO, *P. putida* pWWO; B-DNT, *Burkholderia* sp.DNA. RT (retention time) 11.8 in HPLC and 15.5 in photodiode assay array is identified as 2,4-DNT; RT 10.2 and 13.4 corresponds to Am-DNT; RT 9.2 and 11.6 correspond to TNT; other metabolites are yet to confirm.

¹⁴C-TNT Mass-Balance Studies for Estimating Biodegradation

As described in the previous section, we have tried different approaches to study biotransformation of TNT using HPLC and GC/MS analysis. In this section we describe our attempts to study mineralization of TNT and its metabolites using ¹⁴C-TNT. Mass balance studies of bacteria and fungal cultures indicates that ¹⁴CO₂ trapped in NaOH differed vastly. At our hand we have obtained about 22% of ¹⁴C using the ¹⁴C fungus *P. chrysosporium* whereas we found only 5% using mixed cultures of bacteria. ¹⁴CO₂ evolved in the fungus was vastly accelerated when O₂ was regularly flushed in the growth culture of the fungus. In case of bacterial culture flushing O₂ did not change any rate of ¹⁴CO₂ evolved.

Culture conditions: Bacteria cultures were incubated in basal salt medium, 0.5% sodium acetate and 0.5 µCi of ¹⁴C-TNT (specific activity 21.58 mCi/m mol) and 25 ppm unlabelled TNT. Cultures were grown shaking at 37°C. Fungal culture (*P. chrysosporium*) was prepared by obtaining an aqueous suspension of spores of the fungus from three week old malt extract agar slants (1 ml/ 0.5 absorbance at 650 nm). Suspension of spores were inoculated in low-nitrogen medium and malt extract medium which were grown for six days. On day six of the fungal growth 0.5 µCi (1.1x10⁶ dpm) of ¹⁴C-TNT was added to the cultures. Low-nitrogen medium was used for ligninolytic condition in which peroxidases are produced and malt extract medium was used for non-ligninolytic cultures. Cultures were flushed with oxygen (99.9%) every other day for an incubation period of about 20 days. All controls contained culture medium minus bacterial and fungal inoculum. Culture medium (25 ml) was contained in a 250 ml CO₂ shaking flask (Gledhill, 1975). This flask system and set up was suggested by Dr. Gunninson.

TABLE 7: ¹⁴C-TNT Mass-Balance Studies.

¹⁴ C-Counts	Control (%)	<i>P. putida</i> (pWWO) + Strain B-DNT (in percentage)	Fungus <i>P. chrysosporium</i>	
			By us	Literature*
¹⁴ CO ₂ trapped in NaOH	0	4.05 ± 0.7	21.05	35.4
Water soluble metabolites	4.74 ± 1.5	49.28 ± 3.9	52.91	25.1
Methylene chloride fraction	88.55 ± 2.7	15.01 ± 2.6	8.84	15.7
Bacterial cells	-	10.65 ± 2.2	-	-
Mycelia (fungus)	-	-	2.92	17.3
Wash flask	2.17 ± 2.8	10.26 ± 3.1	1.32	-
Total Radiolabels Recovered	95.46 %	89.25 %	87.04 %	93.5 %

Total ¹⁴C-TNT added in culture media was 800,000 dpm.

*, Taken from Fernando et al., 1990.

Data shown in cells need to confirm using a biooxidizer, which will precisely determine amount of ¹⁴C inside the cells.

VI. SEARCH FOR GENES CAPABLE TO BIOTRANSFORM AND/OR MINERALIZE TNT AND/OR IT'S METABOLITES

Different Approaches for Searching Genes:

We proposed to identify gene(s) capable of degrading TNT. TNT degradation seems to involve a number of genes. For example, four known genes are involved in degrading 2,4-DNT (Suen and Spain, 1993) and 9 more involved in toluene degradation (Franklin et al., 1981). We have obtained soil samples from Army (Dr. H. Tatem, Army, WES, Vicksburg, MS) which contain TNT, as high as 2000 ppm. This soil contain live bacterial cells in such highly contaminated TNT soil indicating that they are capable to use TNT for growth. We approached the problem by (i) making genomic libraries from both bacteria and from the fungus *P. Chrysosporium*. Genomic library of *P. chrysosporium* was a generous gift from Dr. Jacob Reiser (Institute of Biotechnology, Honggerberg, Zurich, Switzerland); and screening recombinant lambda plaques to express TNT degrading genes; (ii) acquiring a number of bacterial and fungal species which either alone or in combination can degrade TNT and Am-DNT, as tested by the most sensitive HPLC analysis; (iii) identifying homologs of some well characterized gene(s), 2,4-DNT using either DNA hybridization screening of the above libraries or by polymerase chain reaction (PCR) technology .

Genomic Libraries : We did not see hybridization between fungal RNA with a probe from a known TNT degradation gene. The fungal genomic library which we obtained from Dr. Reiser may have some TNT degrading gene(s) but the *E. coli* strain which served as a phage host did not grow in TNT media. However, we expect to obtain an *E. coli* strain which can grow in TNT containing media. This will be a good source of host to screen the genomic library again. If some good clones are obtained, we can compare their primary structure with those which we have identified recently by PCR. The shuttle vectors between *E. coli* and *Pseudomonas* were obtained from Dr. Schweizer, Alberta, Canada. The *Pseudomonas* host which we used for this purpose was pRS2015 which does not contain pWWO and thus does not give any background.

Genomic DNA library from *Pseudomonas* and other species: DNAs were isolated from *Pseudomonas* species. We have purified vector from *Pseudomonas putida* (pWWO) since expression of the TNT degrading genes will be optimum if the host is maintained as *Pseudomonas* sp. The DNAs of these species have been digested with restriction enzymes and insertsof 10-15 kb were partially ligated in pWWO vector. Results clearly showed that all strains (supplied by Dr. Gunnison) utilized TNT for growth. Several clones were developed while studying the expression in the shuttle vector followed by transformation to *P. putida* 2015 host. HPLC data showed only biotransformation of TNT to Am-DNT. We also did DNA-sequencing which confirmed using Blast database that they were not in the genes involved in degradation of nitroaromatic compounds.

Role of Mixed Cultures Containing Known Genes:

This approach has been most successful as shown in Figure 4. We have now a *Burkholderia* sp. Culture which can mineralize 2,4-DNT but not TNT. Similarly we have another culture *P. putida* (pWWO) which can mineralize toluene. These culture alone cannot degrade TNT but when mixed almost 80% TNT and Am-DNT disappeared.

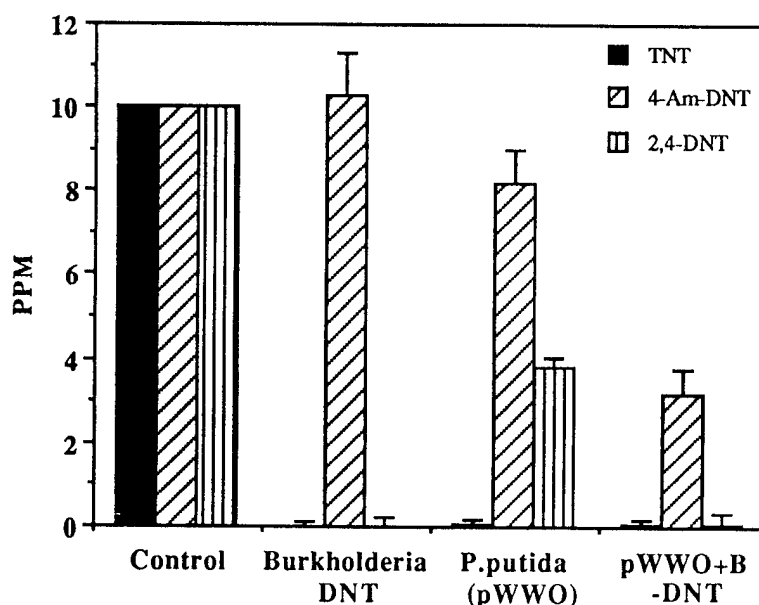


FIGURE 4: Biotransformation of mixed nitroaromatic substances by single and mixed cultures containing 2,4-DNT gene cluster (*Burkholderia* sp.) and TOL gene cluster (*P. putida* pWWO).

Homology Studies of the 2,4-DNT Genes:

The plasmid DNAs of all 10 samples from various bacterial species showed homology to the DNT dioxygenase gene. Homology was also observed in the fungal genomic DNAs. Screening microbes for known 2,4-DNT gene probes and primers based on 2,4-DNT gene sequence such as dioxygenase, monooxygenase, oxygenase and nitroreductase enzyme systems which are in the levels of similarity between the *Pseudomonas* sp. The *dnt A* gene exhibited similarities to other dioxygenase enzymes especially those of the bacteria *Pseudomonas putida*. We have isolated the 6.8 kb size insert from the clone pJS6 (obtained from Jim Spain), was digested with *EcoRI* which revealed that pJS6 has one 14.7 kb, one 1.8 kb, one 0.6 kb and two 2.1 kb fragments. This was used as a probe (α - ^{32}P labeled) and hybridized to ten different bacterial species containing plasmids, genomic DNAs and total RNAs. The fungal (*P. chrysosporium*) genomic DNAs and total RNAs were also hybridized with this probe.

PCR synthesis of a fraction of DNT dioxygenase gene showed that these showed that these sequences present in the *P. chrysosporium* fungal genome. We have sequenced the two PCR products and have compared these sequences in the GenBank. The primers were selected by use of a primer selection software. We tried nine different combinations of primers in order to detect any PCR products. Our results showed that the first set of primers MJ5 (5'-3') and MJ4 (3'-5') which contains 311 bp frag-ment and the second set of primers MJ6 (5'-3') and MJ3 (3'-5') which contains 220 bp fragment size successfully used on *P. chrysosporium* genome DNA as template. The results showed exactly same size using fungal genome but DNA sequences were different from *Pseudomonas* 2,4-DNT open reading frame 3 sequence. Thus indicating a totally different kind of gene. We are presently selecting other primers in other four open reading frames of DNT-A.

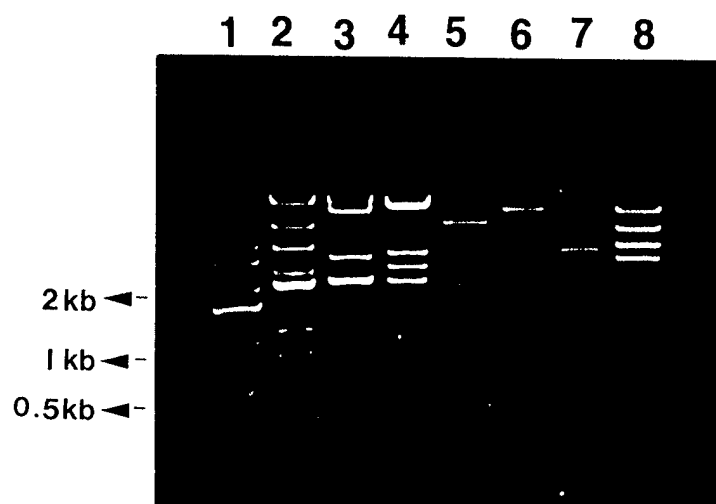


FIGURE 5: Isolation and purification of 2,4-DNT gene cluster probes for rapid screening of the presence of these genes in several other bacterial and fungal cultures. Lane 1, 1 kb STD; lane 2, pJS6 digestion with *EcoRI* + *NsiI*; lane 3, pJS8 digestion with *EcoRI* + *Xho I*; lane 4, pJS19 digestion with *EcoRI*; lane 5, purified 2,4-DNT dioxygenase gene (6.8 kb); lane 6, purified monooxygenase (10 kb); lane 7, purified oxygenase (3.4 kb); lane 8, high mass of the marker.

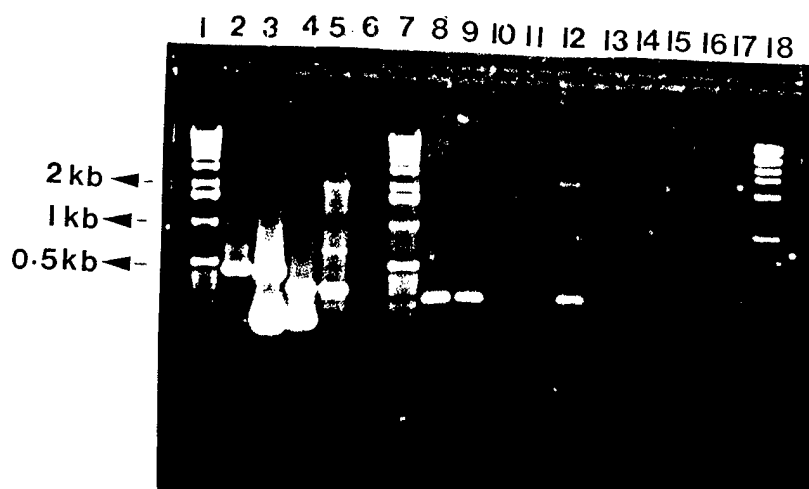


FIGURE 6: Screening for the presence of 2,4-DNT gene homology in the fungus *P. chrysosporium* and several bacterial cultures. Lane 1, 1 kb ladder; lanes 2-5 PCR product using genomic DNA of fungus; lane 6, blank; lane 7, 1 kb ladder; lane 8, genomic DNAs of *E. gergoviae* PCR product; lane 9, *P. aeruginosa* PCR product from genomic DNAs; lane 10, PCR product of genomic DNAs of *K. pneumoniae*; lane 11, PCR product of genomic DNAs of *Xanthomonas maltophilia*; lane 12, PCR product of genomic DNAs of *P. putida* (pWWO); lane 13, *E. gergoviae* of plasmid PCR product; lane 14, *P. aeruginosa* of plasmid PCR product; lane 15, *K. pneumoniae* of plasmid PCR product; lane 16, *X. maltophilia* of plasmid PCR product; lane 18, high mass DNA marker.

Search for Am-DNT Genes from Highly Contaminated Soil With the Explosive TNT:

As mentioned before we have procured and screened several genes involved in the degradation Am-DNT. We have tried to procure two bacterial cultures that degrade Am-DNT as per recent publications (Alvarez et al., 1995; Duque et al., 1993). It is possible that those cultures may be available in future after authors have thoroughly studied them. We have therefore initiated an attempt to isolate Am-DNT degrading culture from a highly (2000 ppm) TNT contaminated soil given to us by U.S. Army (Dr. Tatem). In a preliminary attempt we have had isolated numerous colony forming units (CFU) from soil extract when spread on agar plates containing 50 ppm 2-Am, 4-DNT. It appears that several potential CFU are oxygen sensitive. We are therefore in the process of creating facilities for anaerobic microbiology. We intend to continue this search for Am-DNT gene(s) cluster in both aerobic and anaerobic microbes.

VII. ANCILLARY STUDIES: GENOTOXICITY STUDIES OF TNT METABOLITES.

TNT contaminated soil and underground water imposes serious and widespread environmental pollution. Toxicity of TNT is well established but not much known about TNT metabolites. It was natural therefore for us to ask question about the nature and severity of genotoxicity of TNT metabolites. Using latest state of the art PCR technology we have tested initially three metabolites of TNT such as 2-AM, 4-DNT; 2,4-DNT; and 3-NT by p53 gene mutation. p53 gene mutation has been correlated with carcinogenicity of toxic compounds by several researchers in recent times. We determined the toxicity of TNT metabolites to a hybrid Chinese hamster mouse neuroblastoma NG108 cell line (Dr. M. Nirenberg, NIH, Bethesda, MD). Studies involved: 1) studying cytotoxic effects (using cytotoxic 96™ kit, Promega) of TNT and its metabolites on NG108 cells and determining the lethal dose (or IC₅₀) and optimum exposure time; 2) isolation of RNA and RT-PCR (reverse transcripts polymerase chain reaction) of p53 "mutational hot spots" located in exon 5-9 using amplimers for this region for both control and exposed NG108 cells; 3) DNA sequencing to detect point mutation; 4) isolation of genomic DNA and amplification of p53 intron 5 using primers specific for detection of dinucleotide repeats and loss of heterozygosity (LOH) as described by Jones and Nakamura (1992). Results showed that TNT and its nitroaromatic compounds such as Am-DNT, 4-NT were cytotoxic, among which 4-amino-DNT was the most toxic compound. The IC₅₀ (concentration at which 50% cells die) was at a dose of 100 ppm when exposed for 7 hours. RT-PCR resulted in a 371 bp fragment amplification which is being sequenced for mutation detection. A study for LOH of the p53 DNA using specific primers yielded fragments of 114 bp indicating LOH due to mutation.

Exposing cell to 100 ppm of Am-DNT for 7 hours caused apoptosis as determined by annexin V-FITC and propidium iodide staining of cells as observed under fluorescence microscopy. PCR-based assay of mutational hot spot of p53 tumor suppressor gene derived from cells exposed to 100 ppm of Am-DNT showed point mutation.

VIII. SUMMARY OF ACHIEVEMENTS

Several achievements are made through this initial collaborative project. These are: 1) creation of enormous capability at this HBCU in enhancing this research; 2) extensive studies on identification of TNT biotransformation products in several bacterial species and one fungal species; 3) identification of several genes which are capable of catalyzing degradation of metabolites of TNT in several microbial species, particularly in the fungus *P. chrysosporium*; 4) isolation and DNA sequence of these genes for probe development; and 5) PCR amplification of 2,4-DNT gene using *P. chrysosporium* genomic DNA as template; 6) DNA:DNA hybridization with ³²P labeled 2,4-DNT dioxygenase gene (used as probe) to anneal with *P. chrysosporium* genomic DNAs; and 7) selection of proper combination of mixed genotypes of bacteria for very efficient degradation of TNT.

IX. REFERENCES

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X. PUBLICATIONS

Presentations from this Project (Abstracts Published)

1. S.K. Dutta, Michelle M. Jackson, P.C. Das, J.S. Karns and B. Ghosh. Dept. of Biology, Howard Univ., Washington, D.C. and Dept. of Pesticides, USDA, Beltsville, MD. DNA homology studies can lead to successful bioremediation of toxic chemicals. 8th annual Conf. on hazardous Waste Research, May 24-27, 1993.
2. Dutta, S.K. James Stewart and Li-hua Hou. Dept. of Biol., Howard Univ., Wash. D.C. Microbial biotransformation of 2,4,6-trinitrotoluene (TNT) using *Pseudomonas fluorescens*. 1995 NIGMS Minority Programs Research Symposium, Hyatt Regency Washington Capitol Hill, Wash. D.C., October 11-15., 1995.
3. Li-Hua Hou, Benjamin Dyson Jr. and S.K. Dutta, Dept. of Biol., Howard Univ., Wash. DC. Differential production of TNT biotransformation products by seven bacterial cultures. 1995 NIGMS Minority Programs Research Symposium, Hyatt Regency Washington Capitol Hill, Wash. D.C., October 11-15., 1995.
4. Michelle M. Jackson and S.K. Dutta. Dept. of Biol., Howard Univ., Wash. D.C. Evidence of genes involved in the biotransformation of 2,4,6-trinitrotoluene by the fungus *Phanerochaete chrysosporium*. 1995 NIGMS Minority Programs Research Symposium, Hyatt Regency Washington Capitol Hill, Wash. D.C., October 11-15., 1995.
5. Michelle M. Jackson and S.K. Dutta. Dept. of Biol., Howard Univ., Wash. D.C. Search for gene(s) that biodegrade TNT in *Phanerochaete chrysosporium*. 18th Fungal Genetics Conference, Asilomar Conf. Center, CA. March 21-26, 1995.
6. H.N. Banerjee, M. Verma, M. Ashraf and S.K. Dutta, Dept. of Biol., Howard Univ., Wash. D.C. Cytotoxic effects of trinitrotoluene a(TNT) and its metabolites on NG108 neuroblasoma cells. ASBMB/ASIP/AAI Joint meeting & ASBMB Satellite meetings, New Orleans, LO, June 1-6, 1996.
7. L-H. Hou and S.K. Dutta, Dept. of Biol, Howard Univ., Wash. D.C. TNT biotransformation products differ in several bacterial cultures. 96th ASM general meeting, New Orleans, LO, May 19-23, 1996.
8. L-H. Hou, Adebisi Ajala and S.K. Dutta. Dept. of Biology, Howard Univ., Wash. D.C. Biotransformation of TNT by four strains of *Pseudomonas putida*. 96th National Minority Research Symposium, Miami, FL, November 13-17, 1996.
9. H.N. Banerjee, M. Verma and S.K. Dutta. Dept. of Biol., Howard Univ., Wash. D.C. Alteration of p53 tumor suppresser gene expression by nitroaromatic organic compounds. 96th National Minority Research Symposium, Miami, FL, November 13-17, 1996.

XI. PAPERS IN PREPARATION AS OF 1996.

1. Dutta, S.K., L-H. Hou, Verma, M. and D. Gunnison. TNT biotransformation in several bacteria species: Search for genes.
2. Jackson, M.M., Banerjee, H., Sridhar, R. and Dutta, S.K. Biotransformation of nitroaromatic compounds by the white rot fungus *Phanerochaete chrysosporium* under non-ligninolytic condition.
3. Banerjee, H., Verma, M. and Dutta, S.K. Detection of genotoxicity of 2,4-Am-DNT by identification of dinucleotide repeat polymorphism at the Tp53 locus of MCF7 cells by PCR based assays.
4. Banerjee, H., Verma, M. and Dutta, S.K. Exposure of mammalian cells to 100 ppm of Am-DNT causes cytotoxicity and apoptotic changes.